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Isolation and Characterization of *Mycoplasma conjunctivae* sp. n. from Sheep and Goats with Keratoconjunctivitis

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Fourteen conjunctival strains of mycoplasma were isolated from infected sheep and goats in two separate naturally occurring outbreaks of keratoconjunctivitis (pink-eye). The biological and serological properties and cell protein patterns of these strains are presented in this report. These strains were related to each other and were unrelated to 40 recognized *Mycoplasma* and *Acholeplasma* species and serotypes. We are proposing that this group of mycoplasmas be named *M. conjunctivae*. The role of *M. conjunctivae* in the etiology of keratoconjunctivitis in sheep and goats remains to be determined.

Mycoplasmas cause a number of important diseases in sheep and goats including contagious agalactia and contagious caprine pleuropneumonia (7). In addition, mycoplasmas have been implicated in a variety of other clinical conditions, including conjunctivitis, seen either alone or as a part of a more generalized infection. Although mycoplasmas have been isolated from conjunctival tissues of sheep (12, 14, 23), goats (6, 11, 17), and cattle (9, 13, 14), only limited attempts have been made to identify these strains or to compare them to the recognized species of *Mycoplasma*. However, some of the conjunctival strains isolated from sheep, goats, and cattle have been identified as *M. mycoides* var. *capri* (11), *M. agalactiae* (7), and *M. arginini* (14).

Two naturally occurring outbreaks of keratoconjunctivitis (pink-eye) in two separate sheep and goat herds in Maryland provided an opportunity to examine the occurrence of mycoplasmas in this disease. A total of 14 conjunctival strains were isolated from 8 infected sheep and 6 infected goats. Specimens from noninfected animals were negative. All of the strains had similar biological and serological properties, and they were unrelated to the known species of *Mycoplasma* and *Acholeplasma*. This report presents the characteristics of these strains and suggests that they be considered a new *Mycoplasma* species.

MATERIALS AND METHODS

Media. BBL (8), serum fraction (26), and a modified Hayflick (2) broth and agar media were used.

Specimens. Conjunctival scrapings were obtained from sheep and goats with pink-eye in two separate herds and were cultured for mycopiasma by two of us independently. The specimens were inoculated directly in BBL and Hayflick-type broth and agar media containing antibacterial agents, i.e., thallium acetate (1:4,000 parts) and penicillin G (100 units/ml). The inoculated broth cultures were incubated aerobically for 3 to 7 days and then subcultured in broth and agar media. Agar media were inoculated in duplicate and incubated either aerobically or anaerobically in 5% carbon dioxide in nitrogen (4).

Isolation of strains. The source and origin of the strains isolated and examined are given in Table 1. Eight strains were isolated from eight sheep with pink-eye in a herd located in Cockeysville, Md., and six strains were isolated from six goats with pink-eye in a separate herd located in Poolesville, Md. There was no known contact between the two herds.

Mycoplasmas. Strains HRC581 (sheep) and DBS694 (goat) were initially selected as representative strains and were cloned three times before study. Strain HRC581 was selected later as the type strain and was deposited in the American Type Culture Collection, Rockville, Md. (ATCC 25834). The other species and unclassified strains of *Mycoplasma* and *Acholeplasma* used are given in Table 2. Mycoplasma(s) is used as the trivial term for all *Mycoplasma* and *Acholeplasma* species in the order *Mycoplasmatales* of the class *Mollicutes*.

Test for sterol dependence. The test for sterol dependence was performed by a procedure described previously (20).

Filterability. The filterability of strains HRC581 and DBS694 was determined by a method reported previously (25). In brief, an actively growing broth culture was diluted 10-fold in a phosphate-buffered

TABLE 1. *Isolation and source of Mycoplasma conjunctivae strains*

M. conjunctivae strain	Source of specimen
	Conjunctival scrapings of sheep with pink-eye
HRC581	Sheep no. 581
HRC589	Sheep no. 589
HRC733	Sheep no. 733
HRC781	Sheep no. 781
HRC807	Sheep no. 807
HRC808	Sheep no. 808
HRC814	Sheep no. 814
HRC891	Sheep no. 891
	Conjunctival scrapings of goat with pink-eye
DBS655	Goat no. G237
GBS663	Goat no. G307
DBS665	Goat no. G313
DBS686	Goat no. G242
DBS694	Goat no. G192
DBS695	Goat no. G265

saline solution at *p*H 7.4 with 0.2% gelatin added (PBSG). Filterability of the diluted cultures was determined by use of a Swinney hypodermic adaptor and a series of membrane filters with average porosity sizes of 100, 220, 300, 450, and 800 nm (Millipore Corp., Bedford, Mass.). PBSG provided a more suitable menstruum for test since cultures suspended in PBSG maintained viability and were filtered readily by the use of gentle pressure, thereby avoiding problems associated with high-pressure filtration procedures (15). Mycoplasma titers [colony-forming units (CFU/ml)] were determined for the original cultures and for each of the filtrates by the plate count-dilution procedure.

Biochemical tests. The procedures used to determine dextrose and mannose fermentation, arginine and urea hydrolysis, phosphatase activity, tetrazolium reduction, and serum digestion were reported elsewhere (1, 3). Appropriate mycoplasma cultures were used in each test as positive and negative controls.

Action on erythrocytes. Hemolytic (22) and hemadsorption (16; R. A. Del Giudice and R. Pavia, Bacteriol. Proc., p. 71, 1964) activity for guinea pig and

TABLE 2. *Antisera prepared against the mycoplasmas listed were used in immunofluorescence and growth inhibition tests against Mycoplasma conjunctivae strains HRC581, DBS686, and DBS694*

Species and strain	Species and strain
Primate origin	Murine origin
M. pneumoniae, FH[a, b]	*M. neurolyticum*, type A[a, b]
M. hominis, PG21[a, b]	*M. pulmonis*, ASH[a, b]
Botte[b]	*M. arthriditis*, PG6[a]
M. fermentans, PG18[a, b]	PG27[a, b]
M. salivarium, PG20[a, b]	Canine origin
M. orale, type 1, CH19299[a, b]	*M. spumans*, PG13[a, b]
M. orale, type 2, CH20247[a, b]	*M. canis*, PG14[a, b]
M. orale, type 3, DC333[a, b]	*M. maculosum*, PG15[a, b]
M. lipophilum, MaBy[a, b]	*M. edwardii*, PG24[a, b]
M. primatum[a, b]	*Mycoplasma* sp., HRC689[a]
Mycoplasma sp., Simian HRC291[a, b]	Feline origin
Bovine origin	*M. felis*, 27[a]
M. mycoides var. *mycoides*, Gladysdale[a, b]	CO[b]
M. bovigenitalium, PG11[a, b]	*M. gatae*, Mart[a]
M. bovirhinis, PG43[a, b]	CS[b]
Mycoplasma sp., DBS188 (calf)[b]	*M. feliminutum*, Ben[a]
Ovine and caprine origin	*Mycoplasma* sp., KDA[a]
M. mycoides var. *capri*, PG3[a, c]	Avian origin
M. agalactiae, Agalactiae[a, c]	*M. gallisepticum*, PG31[a]
M. arginini, G230[a, c]	H1344[b]
G506[b]	*M. gallinarum*, PG16[a, b]
Mycoplasma sp., DBS189 (goat)[b]	*M. iners*, PG30[a, b]
Mycoplasma sp., BBL-G145[a]	*M. anatis*, 1340[a, b]
Mycoplasma sp., UM30847[a, b]	*M. meleagridis*, 17529[a, b]
Mycoplasma sp., KS1 (ATCC 15718)[a, b]	Acholeplasma
Mycoplasma sp., 14[a, b]	*A. laidlawii*, PG8[a, b]
Swine origin	PG9[a, b]
M. hyorhinis, GDL[b]	*A. granularum*, BTS39[a, b]
Mycoplasma sp., B3[a, b]	*A. axanthum*, S743[b]

[a] Antisera tested by plate immunofluorescence to *M. conjunctivae* strains HRC581 and DBS694.
[b] Antisera tested by growth inhibition to *M. conjunctivae* strains DBS686 and DBS694.
[c] Antisera obtained from Research Reagents Branch, NIAID, NIH, Bethesda, Md.

sheep erythrocytes was determined by procedures described elsewhere. In brief, hemolytic activity was determined by adding a 3% suspension of washed erythrocytes in 1.5% agar as an overlay to colonies grown on 20% horse serum agar medium.

Polyacrylamide gel electrophoresis. Mycoplasmas grown in serum fraction broth were sedimented, the cell proteins were then solubilized in phenol-acetic acid-water (2:1:0.5, w/v/v), and the extracts were examined in polyacrylamide gels containing 5 M urea and 35% acetic acid (18).

Serological procedures. The plate immunofluorescence (FA) (8), growth inhibition (GI) (5), and metabolic inhibition (MI) (24) tests used were performed essentially as described elsewhere. Slight modifications were made in the GI test, i.e., the serum content of the media was reduced to 5% or was replaced with 1 to 2% serum fraction. Antisera prepared against the antigens listed in Table 2 were produced in either rabbits, goats, or donkeys.

RESULTS

Isolation of strains. Mycoplasmas were isolated by two of us independently from coniunctival scrapings of eight sheep and six goats with pinkeye. Many of the cultures were contaminated with bacteria. Media used for primary isolation of mycoplasma contained penicillin and thallium acetate. The preferred procedure for primary isolation of mycoplasmas was the use of broth media which was subcultured to agar media and incubated in 5% CO_2 in N_2. Some difficulty was encountered in adapting these strains to artificial media. However, after repeated subcultures, most strains grew without difficulty in either an aerobic or an anaerobic atmosphere. Broth cultures produced titers of 10^7 CFU/ml or greater.

Dependence on sterol for growth. All of the strains required serum for growth. The HRC581 and DBS694 strains were examined in more detail for their growth response in various quantities of cholesterol (Table 3). Neither strain exhibited satisfactory growth, as represented by the amounts of cell protein obtained (< 1 mg/100 ml of medium), when grown in a serum-free medium. The addition of albumin or Tween 80 did not improve growth, but cholesterol stimulated growth.

Morphological characteristics: colony morphology. Three colony types were produced. Most colonies had either a fried-egg (Fig. 1) or a granular appearance. Occasionally, colonies grown on Hayflick-type media (2, 8) had elevated centers and a greenish, brownish, or olive color. Some of the low-passage cultures produced colonies which had a small amount of surface growth. The appearance of bacterial forms was not observed when the strains were grown in the absence of penicillin. Film and spots were not produced.

Morphological characteristics: cellular morphology. Unfixed cells from 24- to 48-hr broth cultures of sheep HRC581 and goat DBS694 strains were viewed by phase-contrast microscopy. Both strains exhibited rather uniform individual coccoid elements and small clusters of 2 to 10 coccoid cells joined together by short filaments. The short filaments extending from the center of the cell clusters usually displayed an individual coccoid cell on the end of each filament.

Gram stains of broth cultures revealed gram-negative, pleomorphic spherical, ring-shaped coccobacillary forms. Chains and clumps of cells were seen also. Very few filaments were noted.

Filterability characteristics. Undiluted broth cultures of sheep strain HRC581 contained 1.2×10^8 CFU/ml, and filtrates yielded 7.38×10^5, 4.12×10^5, 1.06×10^4, 20, and 0 CFU/ml for the 800-, 450-, 300-, 220-, and 100-nm filters, respectively. Goat strain DBS694 gave a similar pattern of filterability.

Biochemical characteristics. Identical biochemical reactions were obtained with sheep HRC581 and goat DBS686, DBS694, and DBS695 strains. Positive reactions were produced for dextrose and mannose fermentation and for tetrazolium reduction. Negative reactions were produced for arginine and urea hydrolysis, phosphatase activity, and serum digestion.

Action on erythrocytes. The four strains tested failed to hemadsorb either sheep or guinea pig erythrocytes. All strains produced an α_1-type hemolysis (1, 22) of sheep and guinea pig cells. After prolonged incubation (14 days or more), hemolysis changed from the α_1-type to the β-type.

Electrophoretic patterns of cell proteins. The cell protein patterns of sheep HRC581 and goat DBS686, DBS694, and DBS695 strains were identical to each other (Fig. 2) and were clearly distinct from patterns reported for the other known mycoplasmas (18, 19, 21).

Serological characteristics. The 14 strains listed in Table 1 showed close serological relatedness by the plate immunofluorescence procedure. Each of these strains when tested by FA with antisera prepared against strain HRC581 produced a titer of 1:40 or greater. The serological relatedness of sheep HRC581 and goat DBS686, DBS694, and DBS695 strains was examined further, by using antisera prepared to both sheep HRC581 and goat DBS694. The results of serological studies comparing these four strains by the FA, GI, and MI procedures are presented in Table 4, 5, and 6, respectively. The reciprocal FA titers obtained for each of the four strains were 1:160 or greater when tested with each of the two antisera (Table 4). In addition, the four strains produced reciprocal MI titers of 1:1,280

TABLE 3. *Effect of cholesterol on the growth of Mycoplasma conjunctivae strains in a serum-free medium*

Cholesterol in medium (μg/ml)	Cell protein[a]	
	Sheep HRC 581	Goat DBS 694
0[b]	0.03	0.20
0[c]	0.03	0.12
1.0	3.70	1.51
5.0	3.51	1.36
10.0	3.40	1.96
20.0	4.00	1.64
Control[d]	5.50	3.05

[a] Expressed in milligrams of protein per 100 ml of medium.
[b] Serum-free medium alone.
[c] Serum-free medium with 0.5% albumin and 0.01% Tween 80.
[d] Serum fraction broth (1%).

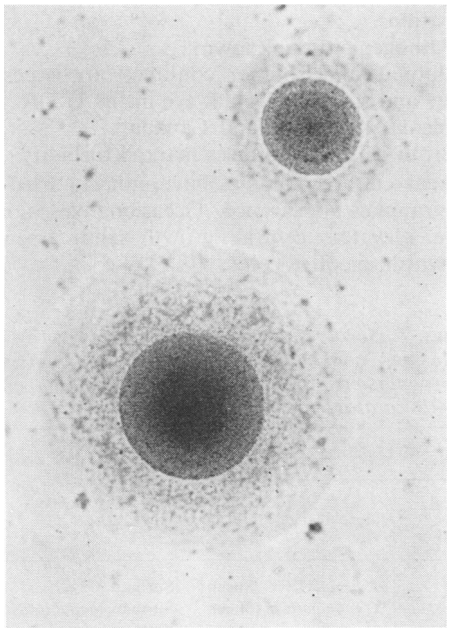

FIG. 1. *Mycoplasma conjunctivae (HRC581). Four-day culture on a modified Hayflick medium (2) stained with Dienes stain. Marker, 100 μ.*

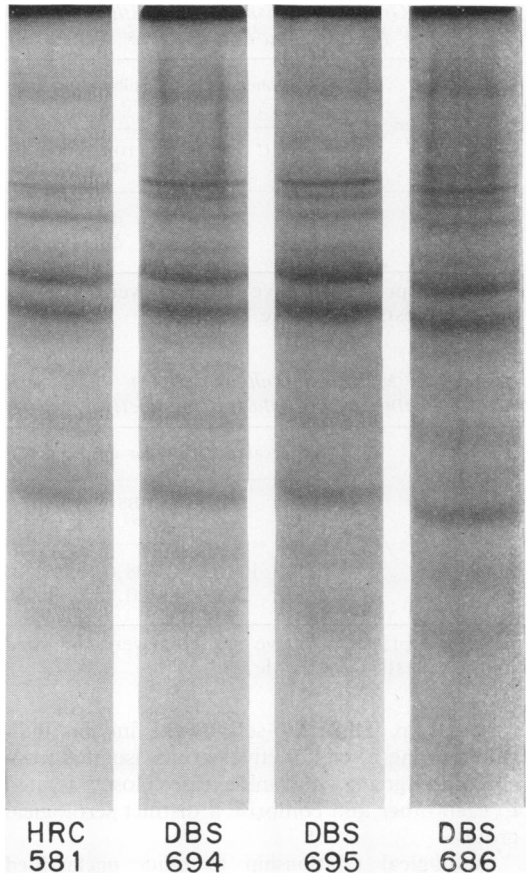

FIG. 2. *Cell protein electrophoretic patterns of Mycoplasma conjunctivae strains.*

TABLE 4. *Plate immunofluorescence tests (FA) with strains of Mycoplasma conjunctivae*[a]

Antisera	Reciprocal of FA titers for antigens to			
	HRC 581	DBS 686	DBS 694	DBS 695
HRC 581	1,280	320	1,280	1,280
DBS 694	160	160	320	160

[a] Appropriate negative controls were included in these tests (see Table 2).

or greater (Table 5), and the growth of these strains was inhibited by the two antisera (Table 6). Appropriate negative control cultures were used in each test. Results of these tests showed that antisera prepared to 40 other mycoplasmas, listed in Table 2, failed either to inhibit the growth or to stain colonies of strains HRC581 or DBS694.

Special emphasis was made on the serological relationships of the conjunctival isolates to *M. mycoides* var. *capri* and *M. agalactiae* because the latter strains share some biochemical properties with our isolates and both of these *Mycoplasma* species have been implicated in sheep and goat conjunctivitis. The results of repeated MI, FA and GI tests utilizing the conjunctival strains as antigens against reference antisera to the two established species failed to show any

TABLE 5. *Growth inhibition tests with strains of Mycoplasma conjunctivae*[a]

Antisera	Zones of growth inhibition (mm) for antigens to			
	HRC 581	DBS 686	DBS 694	DBS 695
HRC 581	5	4	4	4
DBS 694	4	3	4	2

[a] Appropriate negative controls were included in these tests (see Table 2).

TABLE 6. *Metabolic inhibition test (MI) with strains of Mycoplasma conjunctivae*[a]

Antisera	Reciprocal of MI titer for antigens to			
	HRC 581	DBS 685	DBS 694	DBS 695
HRC 581	326,400	10,200	2,608,000	20,400
DBS 694	2,560	2,560	20,480	1,280

[a] Appropriate negative controls were included in these tests (See Table 2).

relationship. Thus, the serological findings indicate that the 14 conjunctival strains isolated from sheep and goats with pink-eye are closely related to each other and comprise a distinct serological group.

Serological relationship to other unclassified mycoplasmas. Table 7 summarizes the data on the relationship of our conjunctival strains HRC581 and DBS694 to unclassified conjunctival strains isolated by other investigators from sheep and cattle with keratoconjunctivitis. Several mycoplasmas isolated from sheep by Surman (23) in Australia were obtained for examination. Two cultures (67R and 83L) were successfully maintained and examined in growth-inhibition and FA procedures. The 67R strain, discussed in Surman's earlier report on sheep pink-eye (23), was shown by us to utilize arginine and was subsequently identified as *M. arginini*. The properties of strain 83L were not included in the earlier study, but this strain was found to be related to our 14 conjunctival strains. Antisera prepared against strains HRC581 and DBS694 inhibited the growth and stained the colonies of strain 83L.

In addition, representative mycoplasmas recovered from sheep pink-eye by Langford (12) were obtained from R. H. Leach (Mycoplasma Reference Laboratory, Colindale, England). One of the strains (M164-69) was partially characterized by Leach (see Langford, reference 12) as a glucose-fermenting strain not related to previously reported sheep or bovine mycoplasmas. The M164-69 strain was shown to be related to our conjunctival strains.

Properties of M. conjunctivae sp. n. In summary, the characteristics of *M. conjunctivae* strains HRC581, DBS694, DBS686, and DBS695 are as follows.

Fermentation: positive for dextrose for all strains; positive for mannose for all strains.

Hydrolysis: negative for arginine and urea for all strains.

Hemolysis: α-type hemolysis of sheep erythrocytes and guinea pig erythrocytes for all strains.

Hemadsorption: negative to sheep erythrocytes and guinea pig erythrocytes for all strains.

Phosphatase activity: negative for all strains.

Tetrazolium reduction (aerobic): positive for all strains.

Serum digestion: negative for all strains.

Film and spots: negative for all strains.

Serum or cholesterol requirement: positive for all strains.

Pathogenicity: unknown.

Habitat and source: conjunctival tissues of goats and sheep with pink-eye in the U.S.A. (see Table 1), Australia, and Canada.

Broth culture: produces marked turbidity.

Agar culture: colonies have either a fried-egg or granular appearance. Occasionally, colonies have elevated central growth and greenish, brownish, or olive color.

TABLE 7. *Serological relationships between Mycoplasma conjunctivae and other unclassified mycoplasmas isolated from sheep and cattle with keratoconjunctivitis*

Mycoplasmas		Host	Investigator and reference
Strain designation	Identification[a]		
67R	*M. arginini*	Sheep	Surman (23)
83L	*M. conjunctivae*	Sheep	Surman (*unpublished data*)
M163-69	*M. arginini*[b]	Sheep	Langford (12) Leach (*personal communication*)
M164-69	*M. conjunctivae*	Sheep	Langford (12) Leach (*personal communication*)
M165-69	Unidentified	Bovine	Langford and Dorward (13) Leach (*personal communication*)

[a] Identification based on growth inhibition and epi-immunofluorescence test procedures.

[b] Also identified earlier as *M. arginini* by R. H. Leach (*personal communication*).

Preferred atmosphere: 5% CO_2 in N_2 for primary isolation. Laboratory-adapted strains grew well in either an aerobic or a CO_2/N_2 atmosphere.

Cellular morphology: gram-negative, pleomorphic, spherical, ring-shaped and coccobacillary forms. Under phase-contrast microscopy, appear coccoid with small clusters of cells joined together by short filament.

Cell protein electrophoretic patterns: *M. conjunctivae* strains have patterns similar to each other but distinct from other established *Mycoplasma* and *Acholeplasma* species in Table 2.

Antigenicity: *M. conjunctivae* strains are related to each other but distinct from other established *Mycoplasma* and *Acholeplasma* species in Table 2 by immunofluorescence and growth inhibition procedures.

Type culture: Strain HRC581 (ATCC 25834) was isolated from conjunctival scrapings of a sheep with pink-eye.

DISCUSSION

This report presents the characterization of the 14 mycoplasmas isolated from sheep and goats with pink-eye. An analysis of the data indicates that these coniunctival strains represent a distinct group of mycoplasmas. These strains were unrelated to those recognized species of *Mycoplasma* [*M. agalactiae* (7) and *M. mycoides* var. *capri* (11)] that have been shown to cause keratoconjunctivitis in sheep and goats.

However, before suggesting a new taxonomic designation for these strains, we deemed it important to compare them to other unclassified mycoplasmas isolated from sheep, goats, and cattle with keratoconjunctivitis. The results obtained with several of these mycoplasmas indicate that at least two strains (83L and M164-69) isolated by other investigators in Australia and Canada were related to our conjunctival strains. Thus, unclassified mycoplasmas, similar in biological and serological properties, have been isolated by several groups of investigators from both sheep and goats with pink-eye in widely separated geographic areas. Surman (23) tentatively suggested that the sheep pink-eye mycoplasmas be named *M. conjunctivae* var. *ovis*. However, it would appear that this subspecies designation does not meet a number of the requirements of the Bacteriological Code (10). Therefore, on the basis of our study, we propose that mycoplasmas with the properties described herein be designated *Mycoplasma conjunctivae* and that the HRC581 strain (ATCC 25834) be considered the type culture.

M. conjunctivae would, therefore, appear to be associated with pink-eye in sheep and goats. Nonetheless, the role of this mycoplasma in the etiology of pink-eye has not been firmly established since Koch's postulates have not been met. Leach (14) reported earlier the isolation of *M. arginini* from the eyes of cattle, sheep, and chamois with keratoconjunctivitis, and this species was also found in some of Surman's (23) and Langford's (12) isolates from pink-eye in sheep. Again, there is no definitive evidence that either *M. arginini* or *M. conjunctivae* is related to the etiology of this disease. Studies currently in progress are designed to establish the role, if any, of *M. conjunctivae* in pink-eye. These studies include attempts to reproduce the disease in animals and also to establish the serological response of animals infected with *M. conjunctivae*.

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